# Individual Human Cell Responses to Low Doses of Chemicals Studied by Synchrotron Infrared Spectromicroscopy

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**ABSTRACT**

Vibrational spectroscopy, when combined with synchrotron radiation-based (SR) microscopy, is a powerful new analytical tool with high spatial resolution for detecting biochemical changes in individual living cells. In contrast to other microscopy methods that require fixing, drying, staining or labeling, SR-FTIR microscopy probes intact living cells providing a composite view of all of the molecular responses and the ability to monitor the responses over time in the same cell. Observed spectral changes include all types of lesions induced in that cell as well as cellular responses to external and internal stresses. These spectral changes combined with other analytical tools may provide a fundamental understanding of the key molecular mechanisms induced in response to stresses created by low-doses of chemicals. In this study we used the high spatial-resolution SR-FTIR vibrational spectromicroscopy as a sensitive analytical tool to detect chemical- and radiation-induced changes in individual human cells. Our preliminary spectral measurements indicate that this technique is sensitive enough to detect changes in nucleic acids and proteins of cells treated with environmentally relevant concentrations of dioxin. This technique has the potential to distinguish changes from exogenous or endogenous oxidative processes. Future development of this technique will allow rapid monitoring of cellular processes such as drug metabolism, early detection of disease, bio-compatibility of implant materials, cellular repair mechanisms, self assembly of cellular apparatus, cell differentiation and fetal development.
Keywords: Infrared spectroscopy, synchrotron, SR-FTIR, dioxin, TCDD, cellular response, spectromicroscopy.

## 1. INTRODUCTION

Conventional non-SR based FTIR spectromicroscopy has been widely used as a diagnostic tool for characterizing the composition and structure of cellular components within intact tissues [1-4], and for measuring tumor tissue responses to therapy [5]. However, the spatial resolution of traditional FTIR spectromicroscopy is limited to ~75 μm with sufficient signal-to-noise [6, 7]. Synchrotron radiation-based FTIR spectromicroscopy, on the contrary, provides several hundred times higher brightness at a diffraction-limited spatial resolution of 10 μm or better, and is therefore a sensitive analytical technique capable of providing molecular information on biological specimens [6-10]. In a recent example, Jamin *et al.* [11] used SR-FTIR to map the distribution of functional groups of biomolecules such as proteins, lipids, and nucleic acids in individual live cells with a spatial resolution of a few microns. In this study we use SR-FTIR spectromicroscopy to measure directly intracellular responses to environmental stimuli.

**Cells and Cell Treatment.** The studies we report on were carried out on normal human lung fibroblast cells, IMR-90, and on human liver derived cancerous cells, HepG2. The IMR-90 cells were grown to confluence and were thusly para-synchronized into the $G_1$ phase. Cells were then scraped and allowed to attach to gold coated microscope slides for SR-FTIR analysis. We then measured cells in the various stages of their growth-cycle. HepG2 cells were obtained from the American Tissue Culture Collection (Rockville, MD). They were maintained in Dulbecco's Minimum Essential Medium supplemented with 10% fetal calf serum, non-essential amino acids, 1 mM L-glutamine, 10 mM N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), and antibiotics. Cells were sub-cultured every 7 days. For TCDD experiments, subconfluent cultures were exposed for 20 hours to $10^{-11}$, $10^{-10}$, and $10^{-9}$ molar of TCDD initially dissolved in pure dimethylsulfoxide

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(DMSO) (Sigma, USA, 99.9% pure). For the control experiment, subconfluent cultures remained in the incubator for 20 hours without exposure to TCDD.

At the end of the 20 hour treatments, cells were either harvested for SR-FTIR analysis or lysed using TRI Reagent™ (Sigma, USA) for RT-PCR studies. Harvesting cells for SR-FTIR analysis was done by trypsinizing and washing twice in ice-cold phosphate buffered saline (PBS). These cell suspensions were kept at 4°C and measured with SR-FTIR within 24 hours. Cells from suspension were pipetted onto a chilled reflecting gold surface for double-pass transmission SR-FTIR analysis. This preparation provides round cells with many separated individuals as shown in Figure 1. As this type of cell line is difficult to synchronize, cells with a diameter of ~20 µm were selected for spectral analysis to narrow the distribution with respect to cell cycle.

## 2. CELL CYCLE AND CELL DEATH

As a first experiment with the synchrotron-based IR technique for single cells we observed the spectrum from the IMR-90 line of normal lung fibroblasts with the possible expectation of seeing differences among cells at different stages in the cell cycle. We observe that cells in the $G_1$, S and mitotic parts of the cycle showed clearly different spectra. Figure 1 shows the 1800 to 900 $cm^{-1}$ region for three single cells. From $G_1$ to mitotic phase cells we observe the amide peaks to approximately double indicating a doubling of the protein, as expected. During S-phase the DNA is undergoing replication and we observe that the DNA/RNA spectral region absorptions increase, followed by a large increase in the dividing cell when a full two copies of the DNA exist. The DNA and RNA spectral bands have considerable overlap, and therefore do not allow an immediate simple interpretation. For example the environment of the DNA is very different in the different phases of the cycle. The binding of the DNA into nucleosomes may, in principle, change the spectrum dramatically. Our cell cycle observations correlate nicely with recently reported results [1, 12].

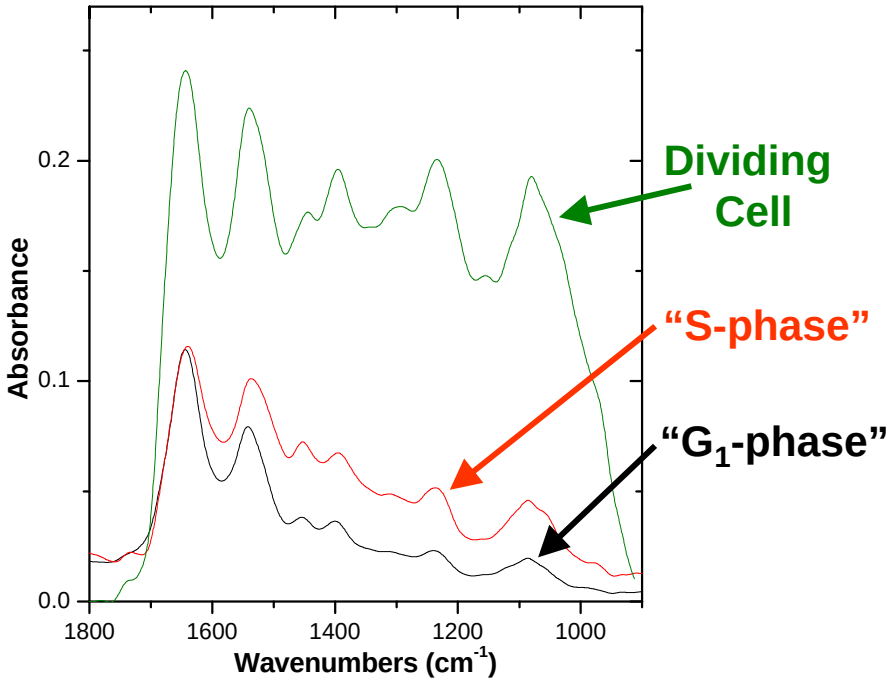


Figure 1. IR spectra of individual cells in different stages of the cell cycle.

Occasionally a cell was measured which exhibited different spectral characteristics near the protein amide I peak. Comparing these spectra to recently presented research on apoptotic cells [13] we see that our spectra indicate that the cells were dying or dead. The spectrum of one such cell is shown in Figure 2 along with the spectrum of a normal living cell. The "dead" cell shows two characteristic spectral signatures indicative of apoptosis, or programmed cell death. First the protein amide I peak's centroid shifts down indicating a change in the overall protein conformational states within the cell. Secondly we observe the appearance of a peak around 1740 $cm^{-1}$. These observations can now be used as signatures of cell death in future studies.

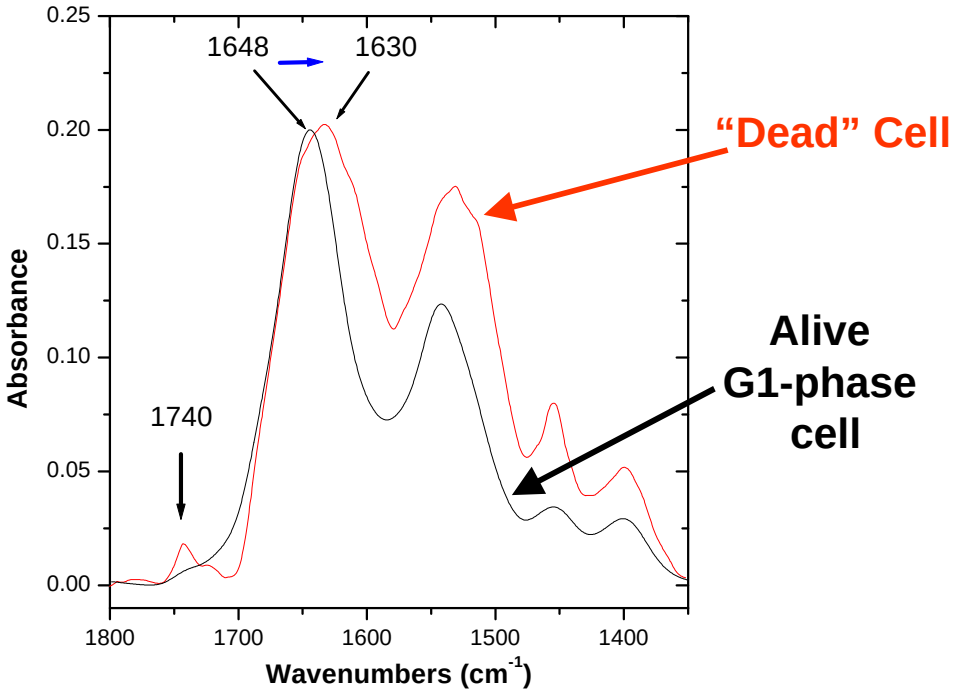


Figure 2. IR spectra comparison of individual live and apoptotic cells.

## 3. HEPG2 CELLS RESPONSES TO TCDD

Exposure to polychlorinated aromatic compounds can lead to various health effects including cancers, alteration of hormone levels, and reproductive defects in animals [14-19] and humans [20-27]. Among this family of pollutants, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is one of the most potent and most studied "man-made" toxins, causing harmful effects at exposure levels hundreds or thousands of times lower than most chemicals of environmental concern [28]. TCDD acts by binding to the aryl hydrocarbon (Ah) receptor [29, 30]. Binding triggers induction of various genes involved in xenobiotic metabolism including the cytochrome P4501A1 (*CYP1A1*) gene [29-33].

The experiments began with exposing HepG2 cells (derived from a human hepatocellular carcinoma) to TCDD at environmentally relevant concentrations. A fraction of the exposed cells were investigated by acquiring SR-FTIR spectra from individual live cells. The remaining cells were analyzed for *CYP1A1* gene expression, using the reverse transcriptase polymerase chain reaction (RT-PCR) technique.

The overall absorbance spectral features of biological materials are well known and our cellular SR-FTIR spectra follow the established pattern. However, by comparing spectra from cells treated with various amounts of TCDD to untreated cells we find significant spectral differences in the magnitude and in some cases the location of peaks at various wavelengths.

Phosphate Bands. Figure 3 shows the IR spectra of unexposed HepG2 cells (solid line) and of cells exposed to different concentrations of TCDD in the phosphate band region. For untreated cells the two phosphate absorption bands [3, 4] at 1236 $cm^{-1}$ (asymmetric phosphate stretching mode $\nu_{as}\ PO_2^-$) and at 1082 $cm^{-1}$ (symmetric phosphate mode $\nu_s\ PO_2^-$) are approximately equal in strength. For TCDD-treated HepG2 cells, the $\nu_{as}\ PO_2^-$ band decreases in intensity while the $\nu_s\ PO_2^-$ band increases by more than a factor of two at the highest TCDD doses studied. The inset to Figure 1 shows the intensity ratio of the $\nu_s\ PO_2^-$ to $\nu_{as}\ PO_2^-$ peaks increases with TCDD concentration. The 1145 to 1190 $cm^{-1}$ region shows a peak that is associated with a C–O vibration[4] that increases in intensity with TCDD concentration.

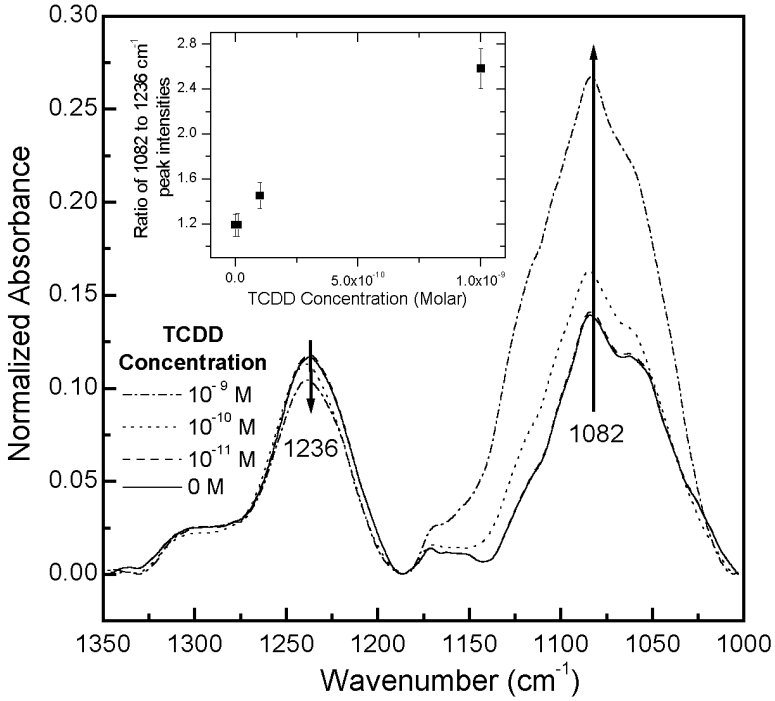


Figure 3. Phosphate IR bands as a function of TCDD dose.

C–H Bands. Spectral absorptions due to hydrocarbon vibrations in lipids, proteins, nucleic acids, sugars, phosphates, among others are found within the 3050-2800 $cm^{-1}$ region. Figure 4 displays the SR-FTIR spectra of unexposed HepG2 cells (solid line) and of cells exposed to different concentrations of TCDD in the C–H stretch region normalized to the peak maximum near 2925$cm^{-1}$. The band near 2853 $cm^{-1}$ is due to the symmetric $CH_2$ stretching of the methylene chains in membrane lipids; the peak at 2925 $cm^{-1}$ is due to the asymmetric $CH_2$ stretch; 2961 $cm^{-1}$ absorption is due to asymmetric stretching of the $CH_3$ methyl groups of both lipids and proteins; and the 2871 $cm^{-1}$ mode is from the symmetric $CH_3$ stretching mode [3, 4]. For TCDD-treated HepG2 cells, the 2853 peak decreases in intensity while the 2961 and 2871 $cm^{-1}$ peaks increase. This indicates that the ratio of the number of methyl groups to that of methylene groups increases as the TCDD concentration increases. The opposite has been found in colorectal cancer tissue analysis.[3, 36] Other authors have proposed that TCDD removes the protection from methylation from certain sites when it binds to the Ah receptor,[37] or increased methylation may down-regulate the expression of the *CYP1A1* gene.[38] Since methylation is so intimately involved with gene inactivation,[39] and we observe a significant increase in the number of methyl groups produced after exposure to TCDD potentially indicating increased methylation, this could explain the tremendous toxicity of TCDD in humans and other animals.

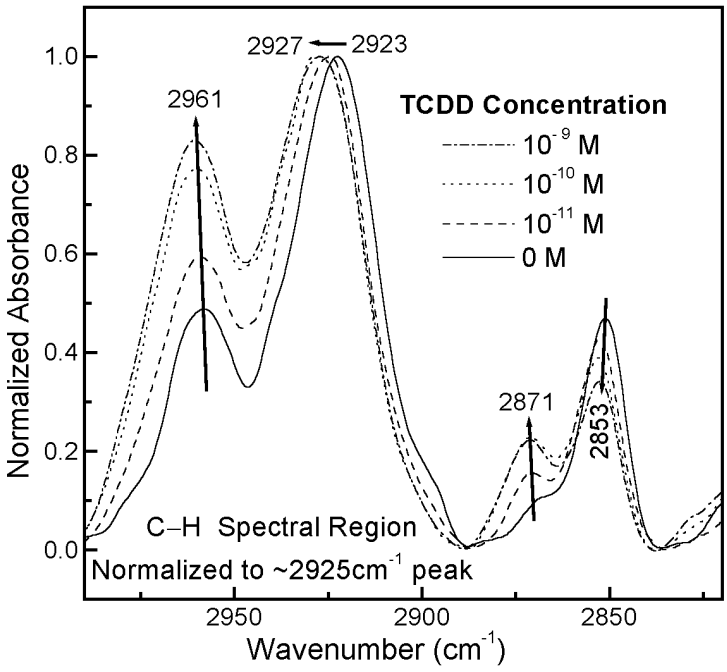


Figure 4. C–H stretch region changes as a function of TCDD dose.

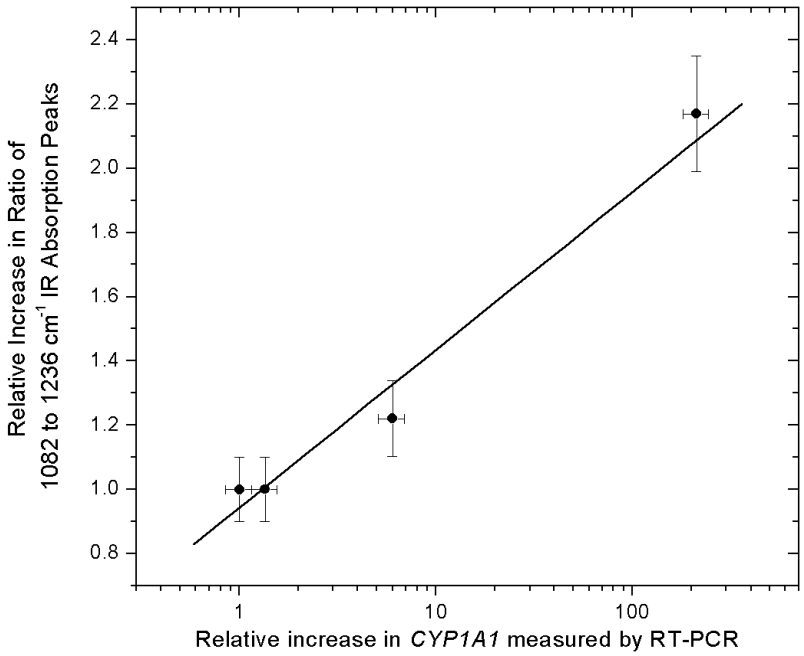


Figure 5. Comparison of SR-FTIR and RT-PCR.

Comparison of SR-FTIR and RT-PCR. RT-PCR was carried out on extracts from the cell cultures of each TCDD exposure. Measured values of *CYP1A1* gene expression were normalized to measured β-actin levels, and finally the relative increase in *CYP1A1* as a function of TCDD was obtained. The relative increase in the ratio of the symmetric to asymmetric phosphate infrared bands with increasing TCDD concentration is compared to the relative increase in *CYP1A1* induction in Figure 5. Error bars for the IR data arise from the fact that we measured at 5 or less cells for each treatment concentration. The solid line in Figure 5 is a weighted linear regression fit to the data. The excellent agreement (with $r^2$ = 0.96) between the two methods indicates that the rapid SR-FTIR spectromicroscopy technique can measure biochemical changes due to the *CYP1A1* expression processes.

# 4. CONCLUSIONS

The SR-FTIR technique is capable of observing subtle changes in individual living cells. Future studies will investigate changes in many different types of cells as well as cellular biochemical processes resulting from a variety of agents. While the infrared spectra of whole cells are quite complex, and it is extremely difficult to assign the changes observed to specific molecular events, the use of cell lines which are defective in a single process or pathway may allow specific mechanisms to be identified in the spectra and studied comprehensively. Once a better understanding of how to interpret IR spectral changes is accomplished, infrared spectromicroscopy may be developed into a rapid and inexpensive diagnostic tool for medical screening applications. The single cell nature of this technique may allow identification of a small number of viable cells in a population that are different from the others, potentially opening new areas of environmental health and biomedical research.

# 5. ACKNOWLEDGEMENTS

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